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# 'APLYSIA' ACETYLCHOLINE RECEPTORS: BLOCKADE BY AND BINDING OF ALPHA -BUNGAROTOXIN

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Armed Forces Radiobiology Research Institute
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13. ABSTRACT

α-Bungarotoxin (αBT) is a snake toxin which in vertebrate systems binds with great specificity to acetylcholine (ACh) receptors. We have studied the effects of αBT on the electrophysiological response to ACh of identifiable cells in the nervous system of the marine mollusc Aplysia and the binding of $^{125}$I-αBT to a ganglionic preparation. Aplysia has three pharmacologically distinct ACh responses and each causes a different conductance change. αBT blocks all three responses of iontophoretically applied ACh. In all cases the inhibition is reversed on washing. Binding of $^{125}$I-αBT to the ganglionic preparation is a saturable process. The dissociation constant of binding calculated from rates of association and dissociation of the toxin-receptor complex was $0.8 \times 10^{-9}$ M. Binding of $^{125}$I-αBT was inhibited by unlabeled toxin, ACh agonists and antagonists as well as by eserine, ouabain, and tetraethylammonium but not by the transmitters serotonin and dopamine.



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# APLYSIA ACETYLCHOLINE RECEPTORS: BLOCKADE BY AND BINDING OF α-BUNGAROTOXIN

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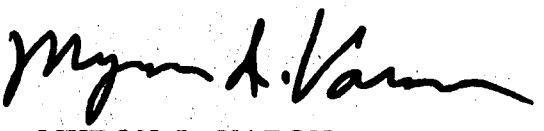

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TABLE OF CONTENTS

## LIST OF FIGURES

# I. INTRODUCTION

The marine mollusc Aplysia has a nervous system containing numerous identifiable giant nerve cells which have proven useful for both electrophysiological and biochemical investigations. Of particular interest is the observation that all of these neurons are sensitive to acetylcholine (ACh).[13] By means of electrophysiological and pharmacological studies, three different types of ACh responses have been identified.[14,30] One is an excitatory depolarizing response resulting from an increase in $Na^+$ conductance and blocked by $10^{-4}$ M d-tubocurarine or hexamethonium.[6,18] The second type of response is mediated by an increase in $Cl^-$ conductance and blocked by d-tubocurarine but not by hexamethonium.[17] At normal resting potentials this response is usually a rapid hyperpolarization. The third response is a late hyperpolarization. This response was originally attributed to ACh activation of an electrogenic $Na^+$ pump since it could be blocked by ouabain or by cooling.[29] More recent studies have attributed a similar response to an increase in $K^+$ conductance which can be blocked selectively by tetraethylammonium, methylxylocholine, or arecoline but not by curare or hexamethonium.[17,18] Because of their difference in pharmacological properties and ionic conductances, each type of response may be associated with a distinct ACh receptor.[18,19]

Recently, several snake venom toxins have been shown to block ACh responses in many vertebrate preparations.[22] One of these toxins, the protein $\alpha$-bungarotoxin ($\alpha$BT), appears to do so by binding specifically and essentially irreversibly to nicotinic ACh receptors.[10,23,27] Hence $\alpha$BT and related toxins have been used to identify and label vertebrate ACh receptors and to assess their biochemical and pharmacological properties.[5,7,11,12,15,26,28,32]

The application of αBT to the study of neurons in Aplysia affords an opportunity to probe ACh responses which not only are pharmacologically distinct but are mediated by fluxes of different ions. In this communication, electrophysiological studies on single identified neurons demonstrated that αBT blocks all three types of ACh responses in Aplysia. In addition, studies on the interaction of radiolabeled αBT with a ganglionic preparation have been used to analyze the properties of toxin binding sites.

## II. METHODS

Experiments were performed on Aplysia dactylomela, collected in the waters around Bermuda, and A. californica, obtained from Pacific Biomarine Supply Company (Venice, California). Ganglia were removed from the animals and, where applicable, cells were identified by the criteria of Frazier et al.[13]

Electrophysiological experiments were performed on ganglia pinned to a paraffin layer in a Lucite chamber. The bath chamber, with a capacity of 5 ml, was perfused with high $Mg^{++}$ (150 mM) seawater to abolish spontaneous synaptic input.[24] Neurons were exposed by a razor slit in the connective tissue capsule and were penetrated with microelectrodes under direct visualization. The recording and current-passing intracellular electrodes were filled with 2 M potassium acetate resulting in a resistance of 0.5 - 4 megohms. The recording procedures were as previously described.[8] Iontophoretic application of ACh was made by passing current pulses (usually $5 \times 10^{-8}$ A) through a glass microelectrode filled with 2 M ACh. Leakage from the pipette was controlled by applying a holding current of opposite polarity ($3 \times 10^{-9}$ A). The electrode was positioned visually near the neuronal soma and the position adjusted until a good ACh response was obtained. No acetylcholinesterase inhibitors were present at

any time. Before the addition of αBT to the recording chamber, perfusion was stopped and the perfusate replaced by high $Mg^{++}$ seawater containing 2 mg/ml bovine serum albumin (BSA) as a carrier protein. αBT was added directly to the bath in a volume not exceeding 100 μl. During exposure to αBT the contents of the bath were constantly recirculated with a peristaltic pump.

Ganglionic preparations were made of pooled cerebral, buccal, pleural, pedal, and visceral ganglia from a number of animals. The ganglia were placed in cold distilled water at a concentration of 10 to 14 ganglia/ml for 10 minutes, homogenized in a ground-glass homogenizer, and centrifuged for 10 minutes at 1000 x g to remove large pieces of debris and cell nuclei. The supernatant was collected and diluted with an equal volume of 2 x concentrated artificial seawater (prepared from Marine Magic, Lampert Kay, Inc., Los Angeles, California) containing 4 mg/ml BSA. This material is defined as the ganglionic preparation. As assayed by labeled bungarotoxin binding, 52 percent of the binding activity remained in the supernatant. All subsequent dilutions and manipulations of the preparation were carried out with Millipore-filtered seawater (type HA filter) containing 2 mg/ml BSA, except where noted. The ganglionic preparation was stored at 4°C and showed less than a 10 percent drop in binding activity on overnight storage.

Diiodo-α-bungarotoxin ($^{125}$I-αBT) with a specific activity of 320-400 Ci/mmole was prepared as previously described.[32] Incubations of the ganglionic preparation with the labeled toxin were made in a polystyrene tube in a total volume of 202 or 222 μl. Incubations were made at 24-25°C. After incubation the mixture was collected on type EGWP Millipore filters. The filters were then washed with BSA containing

seawater (6 volumes of 2 ml) and then with seawater (3 volumes of 4 ml) to remove unbound toxin. The filters were counted in a Packard $\gamma$-counter.

Corrections were made for nonspecific binding of the $^{125}$I-$\alpha$BT to the filters by running parallel samples which lacked the ganglionic preparation. Nonspecific binding of the toxin to the filter was approximately $10^{-15}$ moles.

Protein determinations were made by a modification of the method of Lowry et al.[25]

## III. RESULTS

Electrophysiology. Electrophysiological studies were carried out on single identified neurons of Aplysia to test whether their responses to applied ACh were inhibited by $\alpha$BT. In initial experiments, ACh was added directly to the bath ($10^{-4}$ M final concentration). In appropriate neurons, addition of ACh elicited either depolarizing and/or hyperpolarizing voltage shifts. No responses were observed, however, when $\alpha$BT (approximately $10^{-8}$ M final concentration) had been added to the bath 1 - 2 hours previous to testing. This inhibitory effect of $\alpha$BT was reversed by washing. After perfusion of the bath with seawater for 1 - 2 hours, ACh sensitivity returned to control values.

To obtain a more detailed analysis of this phenomenon, ACh was applied iontophoretically. In addition, the probable ionic mechanism causing the ACh-induced voltage shift was determined for each cell. This procedure was carried out by changing the ionic composition of the seawater (i.e., substituting $Tris^+$ for $Na^+$, see Figure 1) and by directly measuring conductance changes (see Figure 2).

Figure 1 illustrates an experiment performed with R15, a cell with a depolarizing ($Na^+$) response to ACh. Incubation in the presence of $2.5 \times 10^{-7}$ M $\alpha$BT for

15 minutes completely blocked the ACh response of this cell. Within 7 minutes after beginning perfusion of the bath with high $Mg^{++}$ seawater, ACh sensitivity began to return. Recovery of sensitivity was progressive and approached control levels by 90 minutes of washing.

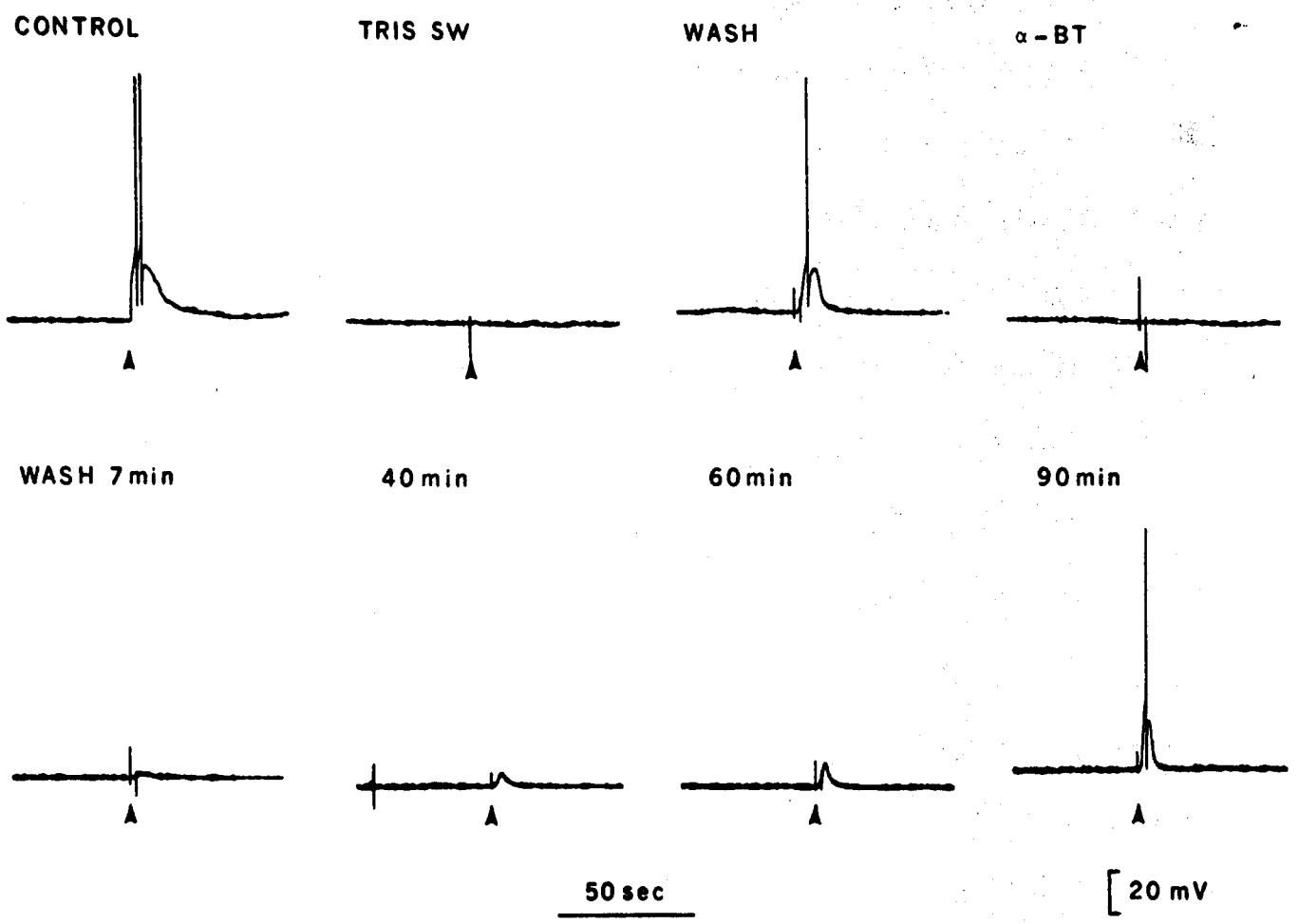



Figure 1. Inhibition by αBT of the $Na^+$-dependent depolarizing response to ACh in R15. ACh was iontophoretically applied in each trace for 3 seconds with a 30 nA current pulse. The onset of the pulse is indicated by an arrow. The first trace shows the control ACh response elicited in slowly flowing seawater. The next trace, labeled TRIS SW, shows the lack of response when equimolar $Tris^+$ was substituted for $Na^+$ in the perfusion fluid and confirms the ionic mechanism of the response. On return to normal seawater (WASH) the response reappears. The trace labeled αBT shows blockade of the response 10 minutes after addition of αBT to the bath ($2.5 \times 10^{-7}$ M final concentration). The lower traces show progressive recovery of ACh sensitivity during rapid perfusion by seawater.

Figure 2 illustrates an experiment performed with L2 a cell known to have both early $Cl^-$ and late $K^+$ ACh responses. At the normal resting potential the late $K^+$ response of this cell is hyperpolarizing while the early $Cl^-$ response is either hyperpolarizing (Figure 2A, Control) or depolarizing (Figure 2A, Wash 30 min). The latter situation results from a progressive accumulation of intracellular $Cl^-$ with time. When the cell is hyperpolarized past the equilibrium potential for $Cl^-$, the two responses are separated since the $Cl^-$ response becomes depolarizing while the $K^+$ response remains hyperpolarizing. With the addition of $1.8 \times 10^{-8}$ M $\alpha$BT to the bath about 2 hours prior to testing, the response to iontophoretically applied ACh was greatly diminished. In other experiments with the same cell but with higher concentrations of toxin, both ACh responses were totally blocked. After perfusion of the bath for 30 minutes, ACh sensitivity was again substantially recovered. These experiments establish that $\alpha$BT blocks all three types of ACh responses in Aplysia and that, in each case, the blockade can be reversed by washing.

Binding properties of the ganglionic preparation. To examine more fully the interaction between the toxin and acetylcholine receptors, experiments were carried out using $^{125}$I-$\alpha$BT and a homogenate preparation of Aplysia ganglia (see Methods). The binding of the labeled toxin was assayed by means of the Millipore filter assay described in the Methods section. With this procedure, toxin bound to membrane fragments was retained by the filter, whereas unbound toxin was not. Over the concentration ranges used in these experiments, the amount of binding observed with a given concentration of toxin was linearly proportional to the amount of ganglionic preparation present.

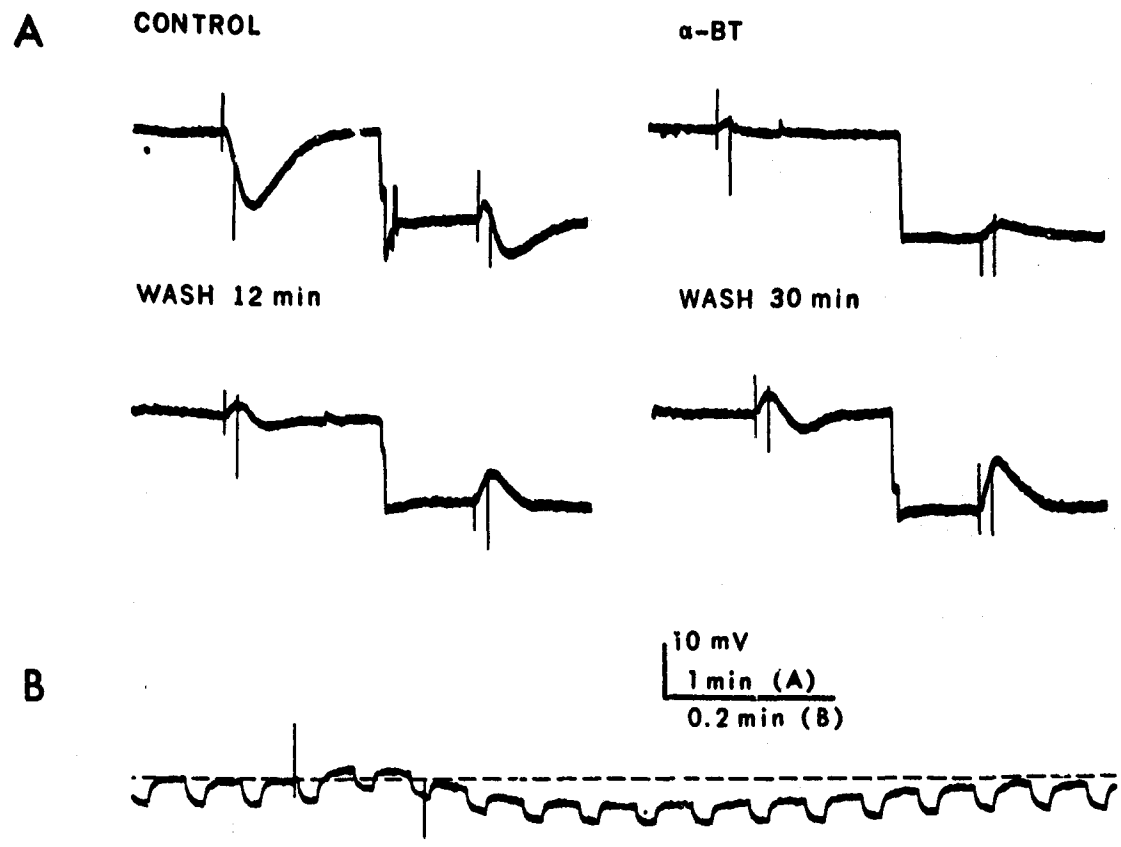



Figure 2. Inhibition by $\alpha$BT of $Cl^-$- and $K^+$-dependent hyperpolarizing responses to ACh. Cell L2 was penetrated by two independent microelectrodes. One was used for recording and the other was used to pass current to vary membrane potential and to pass current pulses for measurement of membrane resistance. The two shock artifact marks indicate on and off of the iontophoretic current for each ACh application. Part A shows the two-component hyperpolarizing responses to iontophoretic ACh (30 nA for 5 sec). The left-hand portion of each trace shows the response at resting potential, whereas at the right the response is elicited when the membrane is hyperpolarized by 15-20 mV. Initially the equilibrium potentials for both $Cl^-$ and $K^+$ are hyperpolarizing from resting potential, but that for $K^+$ is more negative than that for $Cl^-$. As a result, at resting potential the ACh response is negative but at 20 mV hyperpolarized from resting potential the early $Cl^-$ response is depolarizing and the late $K^+$ response is hyperpolarizing (CONTROL). The trace labeled $\alpha$BT illustrates almost complete blockade of response 120 minutes after addition of toxin to a final concentration of $1.8 \times 10^{-8}$ M in the bath. The two traces labeled WASH show recovery of the response upon perfusion with seawater. Now the equilibrium potential for $Cl^-$ has moved depolarizing relative to resting potential and, as a result, the response is biphasic at resting potential and depolarizing when membrane potential is hyperpolarized. Part B documents the increase in conductance during the response shown in the control record with 20 mV hyperpolarization. Constant current pulses (2 nA) cause voltage deflections which decrease in amplitude during both depolarizing and hyperpolarizing components of the response, indicating an increase in membrane conductance.

The binding of $^{125}$I-$\alpha$BT to the ganglionic preparation was saturable. As illustrated in Figure 3, when increasing amounts of the toxin were incubated with equal aliquots of the ganglionic preparation for 1 hour, saturation was reached at toxin concentration of greater than $5 \times 10^{-9}$ M. The saturation level corresponded to approximately 24 pM of toxin bound per mg of protein present in the ganglionic preparation. Half-maximal binding occurred at a toxin concentration of approximately $1 \times 10^{-9}$ M.

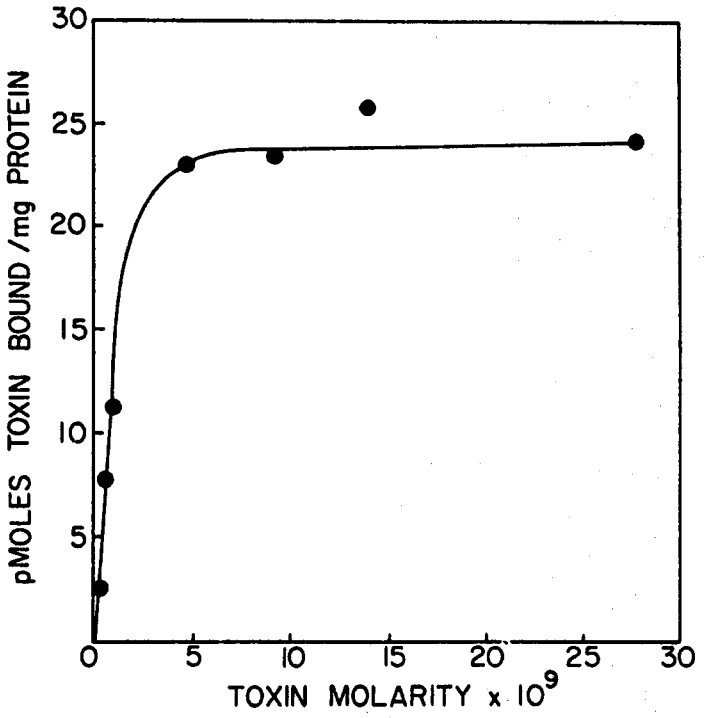



Figure 3. The level of binding of $^{125}$I-$\alpha$BT to the ganglionic preparation after incubation for 1 hour in the presence of various concentrations of the toxin. The conditions for the incubation as well as the methods by which binding was assayed in this and subsequent figures are given in the text. The aliquot of ganglionic preparation used per assay contained 1.4 µg protein.

Experiments were performed to further describe the kinetics of the interaction between the labeled toxin and the ganglionic preparation. Assuming that the toxin binds to a receptor (R), the simplest kinetic model for the interaction would be:

$$^{125}\text{I-}\alpha\text{BT} + \text{R} \underset{\underline{k}_{-1}}{\overset{\underline{k}_1}{\rightleftharpoons}} {}^{125}\text{I-}\alpha\text{BT} \cdot \text{R}$$

where $\underline{k}_1$ and $\underline{k}_{-1}$ are the respective rate constants for the association and dissociation of the toxin-receptor complex $^{125}$I-$\alpha$BT · R. Figure 4 describes the time dependence for binding in the presence of a given concentration of toxin ($3.7 \times 10^{-9}$ M) and ganglionic preparation. Under these conditions, binding reached equilibrium in about 1 hour. If the initial linear rate of binding observed in the time curve is assumed to follow the kinetics for association described in the above model, then $\underline{k}_1$ may be determined from the initial slope of this curve. On this basis, $\underline{k}_1$ was calculated to be $3.3 \times 10^5$ liter moles$^{-1}$ sec$^{-1}$ at 24-25°C.

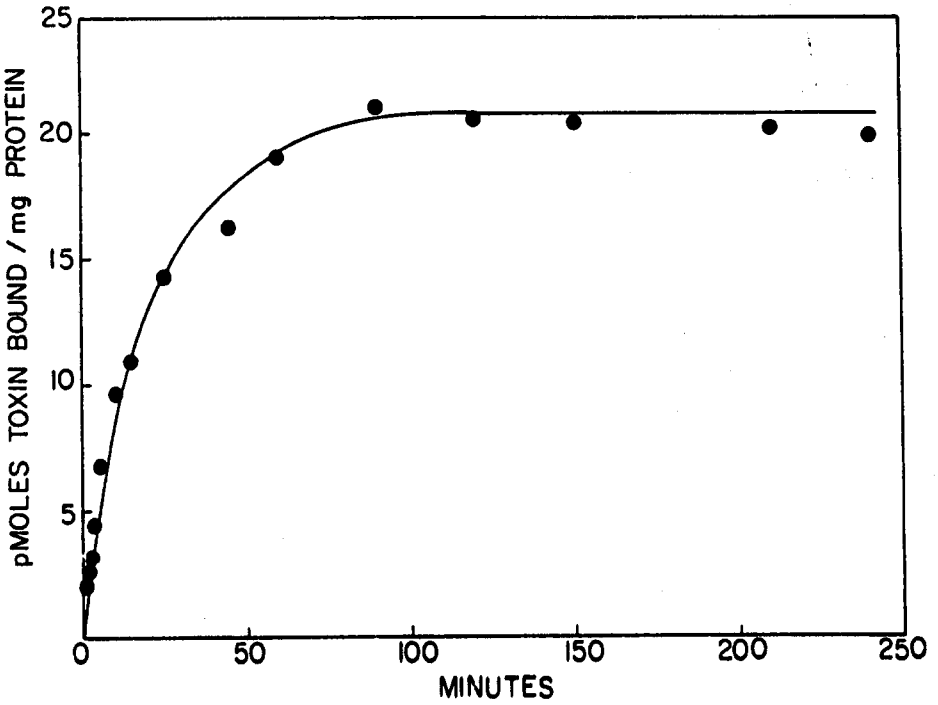



Figure 4. The time dependence of the binding of $^{125}$I-$\alpha$BT to the ganglionic preparation. Equal aliquots of the ganglionic preparation were incubated in the presence of $3.7 \times 10^{-9}$ M labeled toxin for the indicated periods of time.

The dissociation of the toxin-receptor complex was also studied. Identical aliquots of the ganglionic preparation were incubated with $^{125}$I-$\alpha$BT ($3.7 \times 10^{-9}$ M) for 5 minutes. After this time, a 100-fold molar excess of unlabeled toxin was added to each incubation mixture. Under the latter conditions the $^{125}$I-$\alpha$BT receptor complex formed during the initial 5-minute incubation should have been free to dissociate, but would have been competitively inhibited from reassociating by the excess unlabeled toxin. Thus, by measuring the amount of radioactivity bound to the ganglionic preparation at various lengths of time after addition of the unlabeled $\alpha$BT, it was possible to observe the rate of dissociation of the complex. To insure that loss of binding was due to dissociation rather than to instability of the preparation, equal aliquots were incubated for comparable lengths of time without toxin and then with $^{125}$I-$\alpha$BT for an additional 5 minutes. Figure 5 shows that the amount of $^{125}$I-$\alpha$BT bound to the ganglionic preparation decreased with time only in the experimental aliquots. Hence, binding of toxin to the preparation was reversible. If the toxin-receptor dissociation followed the first-order process described by the above kinetic model, a straight line should be obtained if the data in Figure 5 were expressed on a semilog plot. That this was the case is shown in Figure 6. On this basis, $\underline{k}_{-1}$ was determined from Figure 6 to be $2.7 \times 10^{-4}$ sec$^{-1}$ at 24-25°C. The dissociation constant ($K_D = \underline{k}_{-1}/\underline{k}_1$) for the toxin-receptor complex was therefore computed to be $0.8 \times 10^{-9}$ M. The half-life of the complex ($\tau = \ln 2/\underline{k}_{-1}$) was 43 minutes. Note that the latter figure is similar in magnitude to the perfusion period necessary for recovery of ACh sensitivity in the electrophysiological experiments.

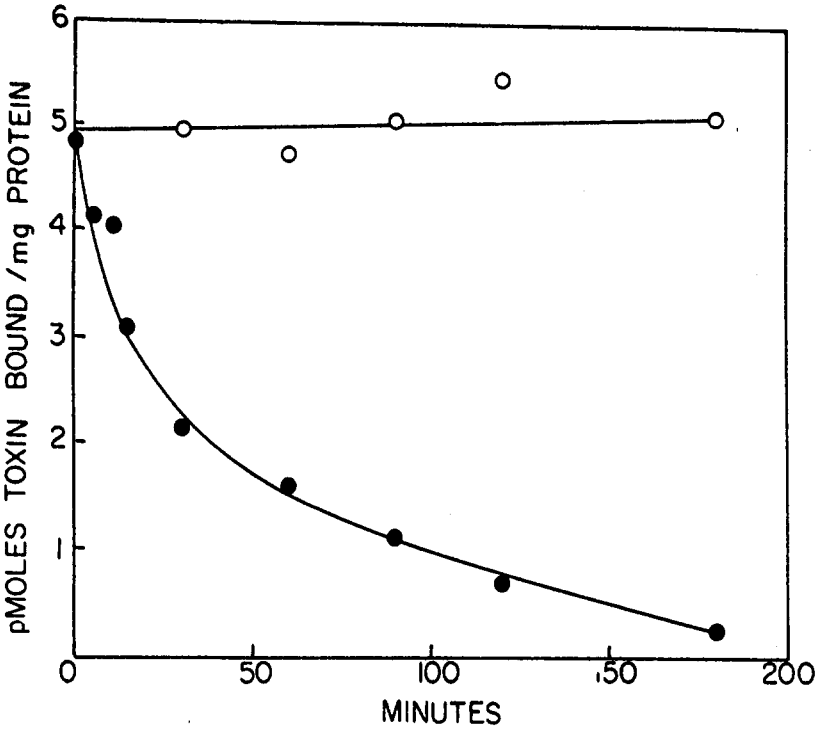



Figure 5. The time dependent dissociation of $^{125}$I-αBT from the ganglionic preparation. A description of this experiment is given in the Results section of the text. The open circles represent control values. The filled circles represent the amount of labeled toxin bound to the preparation after incubation for various lengths of time in the presence of excess unlabeled toxin. The aliquot of ganglionic preparation used per assay contained 1.4 μg protein.

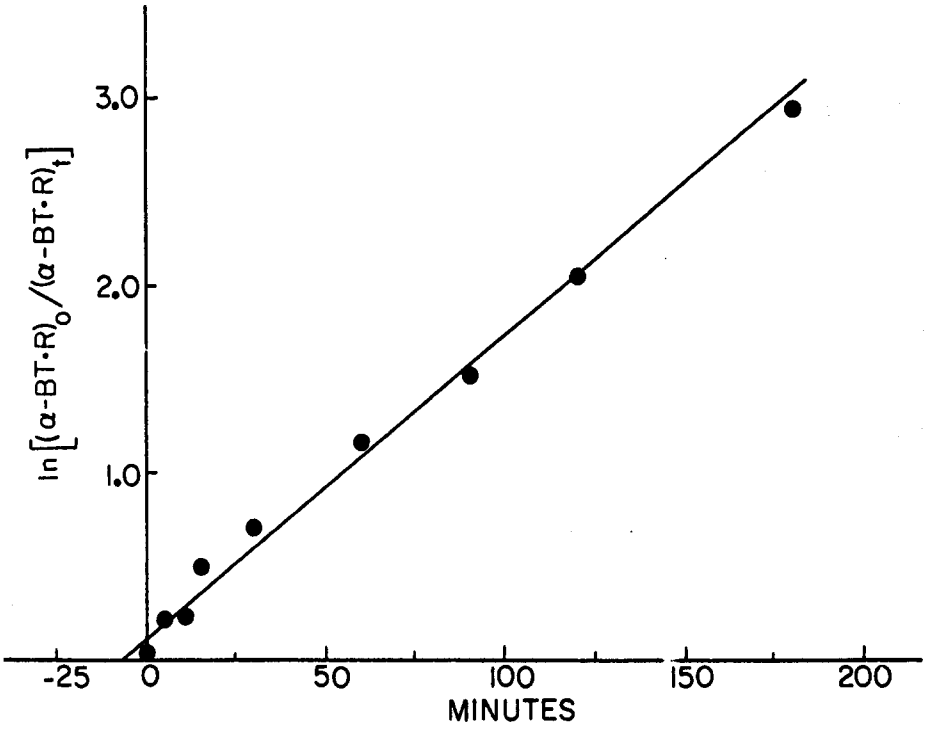



Figure 6. Computation of the apparent first-order rate constant for the dissociation of the receptor-toxin complex (αBT • R) based on the data in Figure 5. For the dissociation of αBT • R the first-order rate constant $\underline{k}_{-1}$ is given as $\ln [(\alpha BT \cdot R)_0 / (\alpha BT \cdot R)_t / t]$ where $[(\alpha BT \cdot R)_0 / (\alpha BT \cdot R)_t]$ is the ratio of the amount of labeled toxin bound at time zero and at time t of incubation with unlabeled toxin.

Inhibition of binding by pharmacological agents. The specificity of toxin binding and the pharmacological properties of the binding sites were investigated. Aliquots of the ganglionic preparation were preincubated for 15 minutes in the presence of various concentrations of a number of agents presumed to bind at or near ACh receptors. Labeled toxin was then added to each mixture (final concentration $3.4 \times 10^{-9}$ M) and binding was assayed after 5 minutes. The time of incubation, the concentrations of $^{125}$I-$\alpha$BT, and the amount of the ganglionic preparation were chosen so that binding in the absence of potential inhibitors was within the initial linear portion of the rate curve. Agents which bind to ACh receptors should protect these sites and thereby depress binding of $^{125}$I-$\alpha$BT.

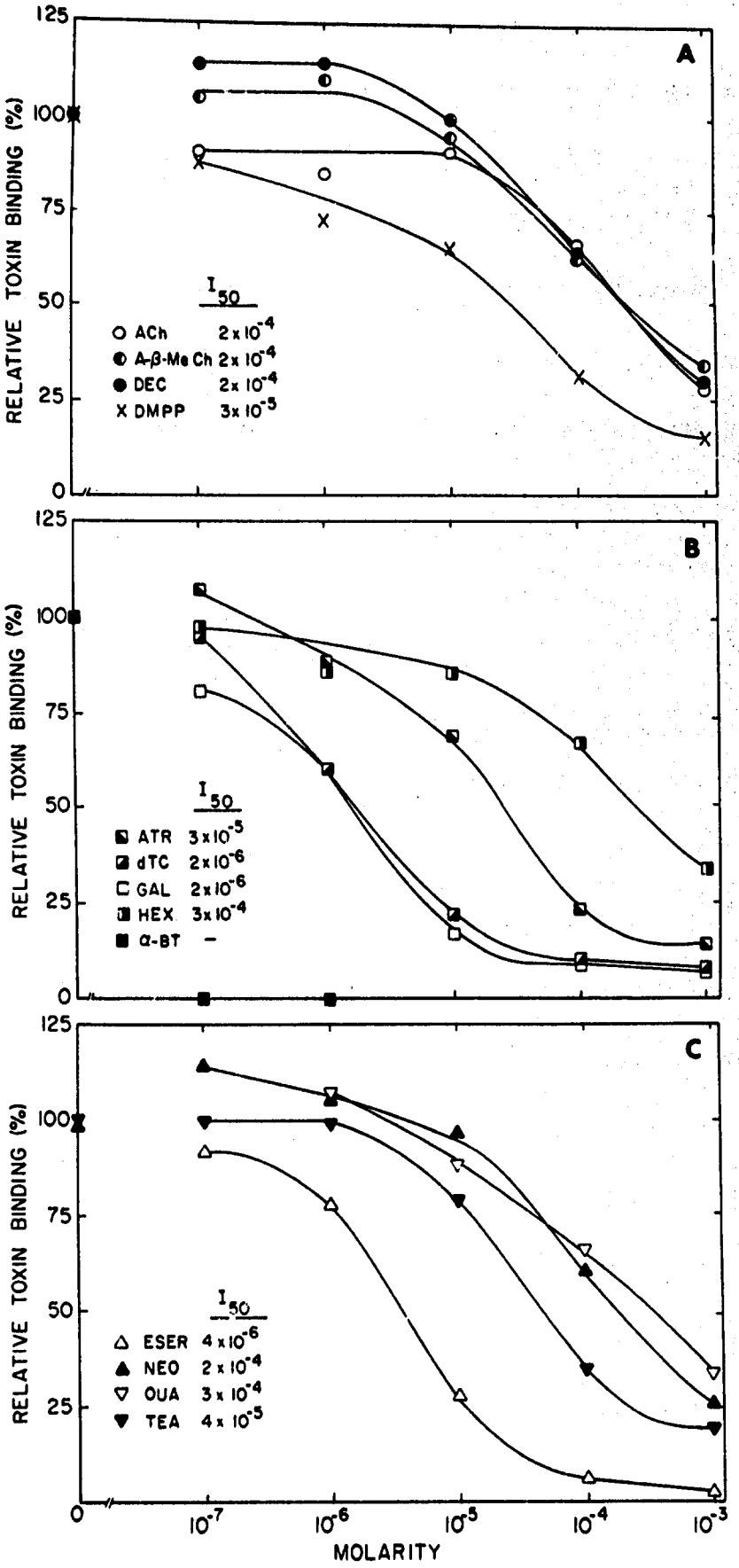
Figure 7A shows the relative binding of $^{125}$I-$\alpha$BT to the ganglionic preparation in the presence of ACh or the other agonists, acetyl-$\beta$-methyl choline (A-$\beta$-Me Ch), decamethonium (DEC) and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP). For ACh the experiment was performed in the presence of $10^{-6}$ M neostigmine to inhibit acetylcholinesterase. All of these agonists depressed $\alpha$BT binding by 50 percent at concentrations ($I_{50}$) ranging from $3 \times 10^{-5}$ to $2 \times 10^{-4}$ M.

Figure 7B shows the inhibition of $\alpha$BT binding by unlabeled toxin as well as by classical antagonists of ACh, including atropine (ATR), d-tubocurarine (dTC), gallamine (GAL) and hexamethonium (HEX). Binding of $^{125}$I-$\alpha$BT was completely blocked by the presence of $10^{-7}$ M unlabeled toxin. Both dTC and GAL were particularly potent inhibitors, having $I_{50}$ values of $2 \times 10^{-6}$ M. ATR ($I_{50} = 3 \times 10^{-5}$ M) and HEX ($I_{50} = 3 \times 10^{-4}$ M) were somewhat less effective. At concentrations of greater than $10^{-4}$ M, dTC and GAL blocked over 90 percent of $^{125}$I-$\alpha$BT binding.

Figure 7C illustrates results obtained with a variety of other drugs which are known to affect the electrophysiological response to ACh but are thought not to compete for the ACh receptor. Eserine (ESER) and neostigmine (NEO) are inhibitors of acetylcholinesterase. Although they may affect the ACh receptor directly in some molluscs,[24] in Aplysia they do not appear to do so (unpublished observations). In contrast to what might have been expected, these drugs blocked binding of $\alpha$BT with ESER having an $I_{50}$ of $4 \times 10^{-6}$ M, and NEO an $I_{50}$ of $2 \times 10^{-4}$ M. Tetraethylammonium (TEA) was studied because of its reported ability to block the synaptic potentials due to a $K^+$ conductance change.[17] Since TEA also blocks $K^+$ conductances not associated with synaptic transmission,[2] it is sometimes assumed that this substance blocks the $K^+$ iontophore rather than ACh receptors. In this regard, TEA, with an $I_{50}$ of $4 \times 10^{-5}$ M, was more effective than expected in blocking binding of labeled $\alpha$BT. Ouabain (OUA) was studied because of its reported but unconfirmed effects on the late hyperpolarizing ACh response.[29] This drug is a potent inhibitor of $Na^+ - K^+$ activated adenosine triphosphatase ($Na^+ - K^+ -$ ATPase).[1] OUA was also somewhat effective in blocking toxin binding with an $I_{50}$ of $3 \times 10^{-4}$ M.

Two putative neurotransmitters, serotonin ($10^{-5}$ to $10^{-3}$ M) and dopamine ($10^{-5}$ to $10^{-4}$ M), did not depress $^{125}$I-$\alpha$BT binding to the ganglionic preparation. These compounds are present in the nervous system of Aplysia.[9] Most Aplysia neurons appear to be sensitive to both serotonin and dopamine.[4,31] Thus the failure of serotonin and dopamine to effect $\alpha$BT binding suggests that the receptors for these two substances are not sensitive to $\alpha$BT and that these compounds in turn do not bind to the $\alpha$BT binding sites.

Figure 7. The effect of various concentrations of different pharmacological agents on the binding of $^{125}$I-$\alpha$BT to the ganglionic preparation. Aliquots of the membrane preparation were preincubated for 15 minutes with the prescribed pharmacological agent; $^{125}$I-$\alpha$BT (final concentration $3.4 \times 10^{-9}$ M) was added and each sample was incubated for an additional 5 minutes before being assayed for binding. Binding is expressed relative to the amount of toxin bound in the absence of potential inhibitors. The concentration of pharmacological agent required to inhibit the binding of labeled toxin by 50 percent ($I_{50}$) is presented in the lower left corner of each part of the figure. Part A presents the results obtained with the cholinergic agonists acetylcholine (ACh), acetyl-$\beta$-methyl choline (A-$\beta$-Me Ch), decamethonium (DEC) and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP). Part B presents the results obtained with the cholinergic antagonists atropine (ATR), d-tubocurarine (dTC), gallamine (GAL), hexamethonium (HEX) and unlabeled toxin ($\alpha$BT). Part C presents the results obtained using the following agents: the antiacetylcholinesterases eserine (ESER) and neostigmine (NEO), the inhibitor of $Na^+$-$K^+$-ATPase ouabain (OUA), and tetraethylammonium (TEA), a drug which affects potassium iontophores and possibly also functions as an antagonist to ACh receptors. The aliquot of ganglionic preparation used per assay contained 2.8 $\mu$g protein.


A
B
C
RELATIVE TOXIN BINDING (%)
125
100
75
50
25
0
I50
ACh 2x10-4
A-β-Me Ch 2x10-4
DEC 2x10-4
DMPP 3x10-5
ATR 3x10-5
dTC 2x10-6
GAL 2x10-6
HEX 3x10-4
α-BT –
ESER 4x10-6
NEO 2x10-4
OUA 3x10-4
TEA 4x10-5
10-7
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MOLARITY

## IV. DISCUSSION

αBT blockade of electrical responses elicited by ACh. The electrophysiological studies presented here show that low concentrations of αBT are capable of blocking each of the three types of ACh responses found on identified neurons of Aplysia. Therefore the toxin appears to be capable of binding to both vertebrate and molluscan ACh receptors. In addition, the blocking effects of the toxin can be reversed for each of the three ACh responses. The rate of reversibility of toxin binding suggests that the recovery of ACh sensitivity with washing is due to dissociation of the toxin from the receptor rather than to the synthesis of new receptors. These findings are in contrast to those reported for vertebrate muscle in which αBT blockage of ACh sensitivity was essentially irreversible.[27]

In the electrophysiological studies, the effects of αBT were studied only for those ACh receptors found on the cell body. These receptors are readily accessible to iontophoretically applied ACh and to the toxin. Although the somal ACh receptors probably have no role in synaptic transmission in Aplysia, the somal receptors of a given cell appear to be identical to functional synaptic receptors found in the neuropile. The conductance changes and the pharmacological sensitivities of the ACh responses are similar for both the somal and true synaptic receptors for a given cell.[19]

Specificity of αBT binding. The interaction between αBT and Aplysia neurons was explored by means of binding experiments performed on a ganglionic homogenate preparation. Of primary concern was whether αBT binds to the ACh receptors and exclusively to the ACh receptors, or, less specifically, to other sites as well. The following points indicate that the binding is specific for ACh receptors.

1. All binding experiments were performed with normal seawater containing a carrier protein (BSA). The high ionic strength of this medium should reduce nonspecific interactions due to charged groups.

2. Binding to the ganglionic preparation was saturable as would be expected for interactions with discrete receptor sites.

3. Specific binding of $^{125}$I-$\alpha$BT to preparations of muscle or other organ tissues of Aplysia was of much lower magnitude than was the binding to the ganglionic preparation (unpublished data).

4. There was a reasonable correlation between the electrophysiological and binding experiments. Toxin concentrations of $10^{-8}$ M blocked electrophysiological ACh responses. The saturation curve (Figure 3), as well as the dissociation constant calculated for the toxin-ganglionic preparation complex, implies half-saturation of the ACh receptors at approximately $10^{-9}$ M. In both electrophysiological and biochemical experiments, binding of the toxin was reversible. The half-life of the toxin-ganglionic preparation complex (43 minutes) was comparable in magnitude to the washing period necessary to regain physiological ACh sensitivity.

5. Although binding was reversible, the $K_D$ value of $0.8 \times 10^{-9}$ M suggests a high affinity process compatible with binding to a specific receptor. In addition, no kinetic evidence was observed which suggested binding to any more than a single receptor site.

6. Binding of $^{125}$I-$\alpha$BT to the ganglionic preparation was depressed in the presence of all the ACh agonists and antagonists tested. Up to 90 percent of the total

binding was inhibited by these substances. Also, a 100-fold molar excess of unlabeled toxin totally blocked binding of $^{125}$I-$\alpha$BT.

7. Not all drugs tested depressed binding. There was no effect from dopamine and serotonin, even though both are charged and presumably are neurotransmitters in Aplysia.[9] It has been reported that the responses to iontophoretically applied dopamine are abolished by curare.[4] In this regard, it appears that the binding of $\alpha$BT to ACh receptors is more specific than that of curare.

In toto, these facts strongly support the view that the binding we have observed is highly specific to ACh receptors.

Level of binding. Saturable binding in the ganglionic preparation reached a value of about 24 pM/mg protein. Although estimates of receptor concentrations in ACh-sensitive tissues vary greatly for different preparations,[28] the level in Aplysia nervous tissue is comparable in magnitude with the highest of these values. In light of the receptor distribution in the Aplysia nervous system, this high magnitude of $\alpha$BT binding is not surprising. Every neuron studied in Aplysia responds to ACh, even though it is not clear that each cell normally receives cholinergic input. Furthermore, electrophysiological evidence suggests that there is widespread presence of functional ACh receptors on both synaptic and nonsynaptic regions of Aplysia neuronal membranes.[13] Since the ganglionic preparation was derived both from cell bodies and the extensive neuropile, it should contain a large amount of ACh-sensitive surface membrane. These considerations suggest not only that the magnitude of $^{125}$I-$\alpha$BT binding observed in the Aplysia is not unreasonable, but that this preparation may serve as a useful source of tissue from which to isolate ACh receptors.

Inhibition of αBT binding by TEA. The extent of inhibition of αBT binding by TEA was greater than expected. TEA can affect synaptic transmission by at least three different mechanisms. It can block permeability changes to potassium when applied to the inside of a squid axon by a direct effect on the $K^+$ channel.[2,3] TEA also causes an increased ACh release from presynaptic terminals,[20,21] presumably as a result of the increased duration of the presynaptic action potential secondary to the inhibition of the $K^+$ conductance. In addition, TEA may act as an ACh antagonist.[33]

In Aplysia, Kehoe[17] has reported that TEA is a specific antagonist for the late ($K^+$) curare-insensitive response at concentrations less than $3 \times 10^{-4}$ M. She concludes that the effect of TEA on this response is a result of a direct antagonism at the ACh receptor and not the iontophore because effects on spike configuration require much greater concentrations. In addition the $K^+$ conductance resulting from activation of specific dopamine receptors is not sensitive to this concentration of TEA. For these reasons it would be expected that TEA would affect only that fraction of ACh receptors which are not sensitive to curare. However, TEA ($10^{-4}$ M) blocked about 70 percent of the αBT binding while the same concentration of curare blocked about 90 percent. From these results it appears that TEA blocks toxin binding to more receptor sites than only those mediating a conductance change to $K^+$.

Inhibition of αBT binding by noncholinergic ligands. The pharmacological agents which were found to affect αBT binding but which are not usually considered agonists or antagonists of ACh are of particular interest. One of the most potent of these drugs was the antiacetylcholinesterase, eserine. Antiacetylcholinesterases would be expected to increase the amplitude and duration of electrical responses to ACh by inhibiting

hydrolysis of the transmitter but would not be expected to affect the ACh receptor directly. However, Tauc and Gerschenfeld[31] have reported that orthodromically evoked synaptic potentials in Aplysia are depressed by eserine, while Levitan and Tauc[24] have reported that eserine selectively blocks depolarizing ACh responses in the mollusc Navanax. In experiments with identified neurons in Aplysia we have found that the presence of eserine always results in an increased amplitude and duration of both D and H responses evoked by iontophoretically applied ACh (unpublished observations). Therefore, eserine does not have a curarizing effect in Aplysia and does not bind to the ACh receptor site. One possibility suggested by these observations and the finding that eserine is a potent inhibitor of toxin binding is that the attachment of eserine to the cholinesterase changes the local environment around the receptor so as to preclude attachment of $\alpha$BT.

The effects of ouabain were investigated because of the early report[29] that this drug blocked the late hyperpolarizing ACh response. Since ouabain binds relatively specifically to $Na^+$-$K^+$-ATPase, the enzyme presumably responsible for active $Na^+$ transport, it was hypothesized that the late ACh response was due to a synaptic activation of an electrogenic $Na^+$ pump.[29] However, the effect of ouabain on this response has not been confirmed and Kehoe[17] attributes the late ACh response to a permeability increase to $K^+$ rather than an electrogenic $Na^+$ pump. These considerations notwithstanding, ouabain did block a portion of the $^{125}$I-$\alpha$BT binding.

Multiplicity of ACh receptors in Aplysia. There is no question that there are three types of physiological responses to ACh in Aplysia. These responses are distinguishable by their pharmacological differences and by their conductance changes.

However, the competitive inhibition experiments and the kinetic analysis have not identified different classes of receptor sites. There are several possible explanations for this observation. In the electrophysiological studies, pharmacological distinctions among the responses have usually been made at only a single concentration of inhibitor. Therefore, the pharmacological distinctions may be a matter of quantitative rather than qualitative differences and such differences might be much more apparent in physiological experiments than in studies on membrare homogenates.

Another explanation for this apparent conflict may be that many pharmacological agents classed as ACh antagonists bind at sites near to but separate from the site of binding of ACh to the receptor.[16] Binding of $\alpha$BT, which is a much larger molecule than ACh, would be more easily blocked by relatively less drastic changes in the local environment than would ACh itself. Thus our assay for interactions using the toxin may be more sensitive for changes in the environment of the receptor than the physiological experiments.

A third possible explanation for our failure to distinguish three types of receptors is that there is such a predominance of one type of receptor that a ganglionic homogenate would appear to contain only that receptor. The most common response electrophysiologically is the $Cl^-$ potential, so if any single receptor predominates it should be this one which is sensitive to curare and gallamine but not hexamethonium, eserine, TEA or ouabain.

Our inability to distinguish three clear classes of receptors is consistent with the hypothesis that the ACh receptor is the same in all responses, and differs only in the iontophore and perhaps other active sites located nearby. We found no distinction

among the receptors on the basis of αBT binding. Each electrophysiological response was sensitive to toxin at about the same concentrations, and the times for inhibition and reversibility were similar. In addition, the kinetic studies performed on the ganglionic preparation showed little indication of other than a single class of binding sites for the toxin.

Further analysis of this question as to the number of receptors and the interaction between receptors, iontophores, and other active nearby sites will be made by studying the binding of $^{125}$I-αBT to single identified cells containing a known receptor type. Preliminary studies have indicated that binding to single cells is of sufficient magnitude to make such experimentation possible.

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